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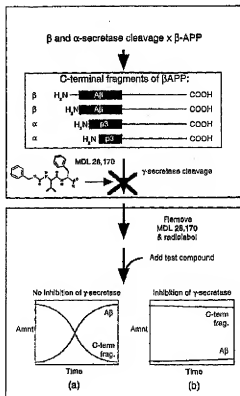
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<b>(21) International Application Number:</b> PCT/US97/16988 <b>(22) International Filing Date:</b> 19 September 1997 (19.09.97) <b>(30) Priority Data:</b> 08/726,524      7 October 1996 (07.10.96)      US <b>(71) Applicant (for all designated States except US):</b> SCIOS INC. [US/US]; 2450 Bayshore Parkway, Mountain View, CA 94043 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CORDELL, Barbara [US/US]; 4051 Ben Lomond Drive, Palo Alto, CA 94306 (US); HIGAKI, Jeffrey, N. [US/US]; 2111 Windrose Place, Mountain View, CA 94043 (US). <b>(74) Agents:</b> SHEARER, Peter, R. et al.; Scios Inc., 2450 Bayshore Parkway, Mountain View, CA 94043 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GR, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, NG, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

**(54) Title:** METHOD TO IDENTIFY DIRECT INHIBITORS OF THE BETA-AMYLOID FORMING ENZYME GAMMA-SECRETASE**(57) Abstract**

A method for identifying direct inhibitors of  $\gamma$ -secretase is described. A cell line expressing  $\beta$ -APP is cultured in contact with a compound known to inhibit  $\gamma$ -secretase activity, thereby causing accumulation of  $\beta$ -APP carboxyl-terminal fragments in the cell. The known  $\gamma$ -secretase inhibiting compound is removed and replaced with a test substance. The direct  $\gamma$ -secretase inhibitory activity of the test substance is determined by quantifying the amount of  $\beta$ -APP carboxyl-terminal fragments in the cells and/or quantifying the amount of  $\beta$ -amyloid peptide in the culture medium over time.



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## METHOD TO IDENTIFY DIRECT INHIBITORS OF THE BETA-AMYLOID FORMING ENZYME GAMMA-SECRETASE

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## Field of the Invention

The present invention relates to assays useful for identifying agents for the  
10 treatment of Alzheimer's disease.

## Background

Alzheimer's disease is a common age-related brain degenerative disease. The  
15 disease is characterized by progressive dementia together with the presence of  
characteristic neuropathological features. The formation of  $\beta$ -amyloid deposits or  
plaques is a hallmark and diagnostic feature of Alzheimer's disease (Khachaturian (1985)  
Arch. Neurol. 42:1097-1105). A significant body of evidence suggests that the process of  
 $\beta$ -amyloid formation and deposition is directly linked to the development of this disease.  
20 For example, individuals with mutations in the gene encoding the  $\beta$ -amyloid precursor  
protein ( $\beta$ -APP) invariably develop Alzheimer's disease (Goate et al. (1991) Nature  
353:844-846; Mullan et al. (1992) Nature Genet. 1:345-347; Murrell et al. (1991)  
Science 254:97-99; Van Broeckhoven (1995) Eur. Neurol. 35:8-19).

25 The  $\beta$ -amyloid peptide is a 39-43 amino acid protein (Glenner et al. (1984)  
Biochem. Biophys. Res. Commun. 120:885-890; Masters et al. (1985) Proc. Natl. Acad.  
Sci. USA 82:4245-4249) which is capable of forming  $\beta$ -pleated sheet polymers. These

- polymeric fibrils are subsequently deposited in the brain parenchyma or in the cerebrovasculature of the Alzheimer's disease victim. The  $\beta$ -amyloid peptide is derived from a larger Type I membrane spanning protein,  $\beta$ -APP, which has several alternatively spliced transcripts (Kang et al. (1987) Nature 325:530-532; Ponte et al. (1988) Nature 331:525-527; Tanzi et al. (1988) Nature 331:528-530; Kitaguchi et al. (1988) Nature 331: 530-532; de Savage et al. (1989) Science 245:651-653). The differentially spliced transcripts give rise to  $\beta$ -APP of 695, 714, 751, and 770 amino acids. The biological function of  $\beta$ -APP is not well understood although it appears to function in cell to cell contact, cell survival, and cell proliferation (Schubert et al. (1989) Neuron 3:689-694; Saitoh et al. (1989) Cell 58:615-622; Chen et al. (1991) Neurosci. Lett. 125:223-251; Mattson et al. (1993) Neuron 10:243-254).

- A secreted form of  $\beta$ -APP is normally generated by proteolytic cleavage (Weidemann et al. (1989) Cell 57:115-126). This proteolytic cleavage occurs within the  $\beta$ -amyloid domain precluding  $\beta$ -amyloid formation (Esch et al. (1990) Science 248:1122-1124; Sisodia et al. (1990) Science 248:492-495). As a result of the cleavage, the bulk of  $\beta$ -APP is released from the cell and a carboxyl-terminal fragment of ~8 kDa remains bound to the cell membrane. The enzyme(s) responsible for this non-amyloidogenic processing of  $\beta$ -APP is termed  $\alpha$ -secretase.

- The formation of  $\beta$ -amyloid peptide is a normal physiological process. The peptide has been found to be naturally produced by cultured cells *in vitro* (Haass et al. (1992) Nature 359:322-325; Seubert et al. (1992) Nature 359:325-327; Shoji et al. (1992) Science 258:126-129; and *in vivo* (Seubert et al. (1992) Nature 359:325-327; Vigo-Pelfrey et al. (1993) J. Neurochem. 61:1965-1968; Tabaton et al. (1994) Biochem. Biophys. Res. Commun. 200:1598-1603; Teller et al. (1996) Nature Med. 2:93-95). The  $\beta$ -amyloid peptide appears to be a degradation by-product of intracellular catabolism of

- the non-secreted form of  $\beta$ -APP (Higaki et al. (1995) Neuron 14:651-659) and inhibiting its formation has no apparent deleterious consequences *in vitro*. There are two proteolytic processing steps required to produce the  $\beta$ -amyloid peptide: one produces the amino-terminus of the peptide mediated by an unidentified enzyme(s) referred to as
- 5  $\beta$ -secretase, the second forms the carboxyl-terminus of the peptide which is generated by an unidentified enzyme(s) termed  $\gamma$ -secretase. The  $\beta$ -secretase processing of  $\beta$ -APP occurs first as evidenced by the intracellular production of  $\beta$ -APP carboxyl-terminal fragments harboring the intact  $\beta$ -amyloid domain which have been demonstrated to be the direct precursors to the  $\beta$ -amyloid peptide (Higaki et al. (1995) Neuron 14:651-659;
- 10 Golde et al. (1992) Science 255:726-730). These ~10-12 kDa  $\beta$ -APP carboxyl-terminal fragments are subsequently processed by  $\gamma$ -secretase to yield  $\beta$ -amyloid. In addition, the  $\alpha$ -secretase, non-amyloidogenic  $\beta$ -APP carboxyl-terminal fragments are processed together with the amyloidogenic fragments by  $\gamma$ -secretase resulting in a ~3 kDa partial  $\beta$ -amyloid peptide (roughly spanning residues 17 to 39-43 of the  $\beta$ -amyloid domain).

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- Attention has been directed to inhibiting the processing of  $\beta$ -APP into  $\beta$ -amyloid peptide as an approach to novel therapeutic development for Alzheimer's disease. Neither the  $\beta$ - nor  $\gamma$ -secretase processing enzymes have been identified or purified. No assay exists which contains pure  $\beta$ -APP and pure  $\beta$ -amyloid forming enzymes. Intact
- 20 cultured cells provide a source of  $\beta$ -amyloid peptide. Consequently, cultured cells are typically employed to screen and identify agents which block  $\beta$ -amyloid formation. However, active agents are likely to affect  $\beta$ -amyloid formation by a number of different mechanisms such as inhibition of  $\beta$ -secretase, inhibition of  $\gamma$ -secretase, alteration of intracellular compartments in which  $\beta$ -amyloid processing occurs (Busciglio et al. (1993)
- 25 Proc. Natl. Acad. Sci. USA 90:2092-2096; Higaki et al. (1995) Neuron 14:651-659), inhibition of  $\beta$ -APP transcription or translation, inhibition in  $\beta$ -APP intracellular transport; as well as may other undefined processes. It is, therefore, desirable in the

absence of pure  $\beta$ -amyloid processing enzymes to have an assay which can distinguish among these many mechanisms of  $\beta$ -amyloid inhibition which can occur in an intact cell. Currently, there is no method to distinguish agents which directly inhibit  $\gamma$ -secretase activity from those which inhibit  $\gamma$ -secretase activity by indirect mechanisms such as

5 alteration of compartments in which  $\beta$ -amyloid processing occurs. Direct acting  $\gamma$ -secretase inhibitors are preferred as potential therapeutics for the treatment of Alzheimer's disease over agents which perturb normal  $\beta$ -APP biosynthesis and activity or which perturb general cellular compartments and functions. Accordingly, it would be highly desirable to have a method for identifying agents which directly inhibit  $\gamma$ -secretase

10 activity.

### Summary of the Invention

The present invention provides a method for identifying direct acting  $\gamma$ -secretase

15 inhibitors of  $\beta$ -amyloid formation using intact or semi-intact cultured cells. The method can be used to identify preferred therapeutic compounds or compositions for the treatment of Alzheimer's disease. The method of the invention comprises the steps of:

- (A) culturing a cell line which expresses a DNA sequence encoding  $\beta$ -APP and which is capable of producing  $\beta$ -amyloid peptide in contact with a substrate accumulating
- 20 compound which causes the accumulation of  $\beta$ -APP carboxyl-terminal fragments;
- (B) removing the substrate accumulating compound;
- (C) contacting the cell line with a test substance; and
- 25 (D) determining the  $\gamma$ -secretase inhibitory activity of the test substance by quantifying the conversion of carboxyl-terminal fragments of  $\beta$ -APP to  $\beta$ -amyloid peptide over time.

The method of the invention can be carried out using a culture of intact cells or semi-intact cells prepared by standard methods such as hypotonic buffer treatment and mechanical shearing or with cells whose cell membranes have been rendered permeable using known agents such as digitonin or non-ionic detergents.

Various techniques may be employed to quantify  $\beta$ -APP carboxyl-terminal fragments and/or  $\beta$ -amyloid peptide. Such methods may include immunoprecipitation or Western blot coupled with polyacrylamide gel electrophoretic separation of protein, radioimmunoassay, or immunodisplacement assay. In a preferred embodiment immunoprecipitation followed by gel electrophoresis is used to quantify  $\beta$ -APP accumulated fragments and  $\beta$ -amyloid peptide, both of which are metabolically radiolabeled during the assay procedure. The preferred embodiment is to use  $^{35}\text{S}$ -methionine radiolabeling but alternative embodiments may use  $^3\text{H}$ - or  $^{14}\text{C}$ -amino acid radiolabeling.

### Brief Description of the Figures

Figure 1 is a schematic illustration of the assay method of the invention.

Figure 2 is an autoradiograph of polyacrylamide gels showing the ability of substrate accumulating compound to cause the accumulation of  $\beta$ -APP carboxyl-terminal fragments and inhibit the formation of  $\beta$ -amyloid peptide.

Figure 3 is a graphical representation of the analysis of polyacrylamide gels for a test substance which is a direct  $\gamma$ -secretase  $\beta$ -amyloid inhibitor.

Figure 4 is a graphical representation of the analysis of polyacrylamide gels for a test substance which is inactive as a direct  $\gamma$ -secretase  $\beta$ -amyloid inhibitor.

### Detailed Description of the Invention

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As indicated above, the invention involves a method of identifying agents capable of inhibiting the formation of the carboxyl-terminus of the  $\beta$ -amyloid peptide by directly inhibiting the activity of  $\gamma$ -secretase. Such agents can be used to intervene in the process of Alzheimer's disease amyloidosis.

10

As used herein, the term " $\beta$ -amyloid peptide or protein" refers to any of the  $\beta$ -amyloid species of ~4 kDa known to have several different amino-termini and heterogenous carboxyl-termini based on characterization of the peptide from Alzheimer's disease tissue and from cultured cells (Glennner and Wong (1984) Biochem. Biophys. Res. Commun. 120:885-890; Joachim et al. (1988) Brain Res. 474:100-111; Prelli et al. (1988) J. Neurochem. 51:648-651; Mori et al. (1992) J. Biol. Chem. 267:17082-17806; Seubert et al. (1992) Nature 359:325-327; Naslund et al. (1994) Proc. Natl. Acad. Sci. USA 91:8378-8382; Roher et al. (1993) Proc. Natl. Acad. Sci. USA 90:10836-10840; Busciglio et al. (1993) Proc. Natl. Acad. Sci. USA 90:2092-2096; Haass et al. (1992) Nature 359:322-325). Specifically, with regard to the carboxyl-termini, the  $\beta$ -amyloid peptide has been shown to end at position 39, 40, 41, 42, 43, or 44 where position 1 is the aspartate of the  $\beta$ -amyloid sequence as defined by Glennner and Wong (1984) Biochem. Biophys. Res. Commun. 120:885-890. This assay method applies to all  $\beta$ -amyloid species with various carboxyl-termini.

25

The term " $\beta$ -amyloid precursor protein" or " $\beta$ -APP" refers to any of the differentially spliced isoforms of this protein including the 695, 714, 751, and 770 amino



- acid isoforms (Kang et al. (1987) Nature 325:530-532; Ponte et al. (1988) Nature 331:525-527; Tanzi et al. (1988) Nature 331:528-530; Kitaguchi et al. (1988) Nature 331: 530-532; de Savage et al. (1989) Science 245:651-653). This term also applies to naturally occurring human mutants of  $\beta$ -APP (Goate et al. (1991) Nature 353:844-846;
- 5 Mullan et al. (1992) Nature Genet. 1:345-347; Murrell et al. (1991) Science 254:97-99; Van Broeckhoven (1995) Eur. Neurol. 35:8-19); to wild-type or any mutant  $\beta$ -APP produced by cultured cells using recombinant DNA methodology; and to natural or artificial derivatives of  $\beta$ -APP which are capable of generating  $\beta$ -amyloid.

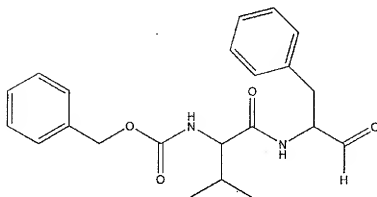
- 10 The term " $\beta$ -APP carboxyl-terminal fragments" refers to carboxyl-terminal derivatives of  $\beta$ -APP which result from  $\alpha$ -secretase and/or  $\beta$ -secretase processing and which generate fragments bearing the  $\beta$ -amyloid domain at or near the amino-terminus of the fragment or partial  $\beta$ -amyloid domain sequences.

- 15 The term "substrate accumulating compound" refers to an agent which has been shown, when contacted with a cell line expressing  $\beta$ -APP, to cause the intracellular accumulation of  $\beta$ -APP carboxyl-terminal fragments, which simultaneously blocks  $\beta$ -amyloid formation, and the activity of which can be reversed upon removal of the compound. Examples of such agents are ammonium chloride, monensin and the
- 20 compound MDL 28,170 (Higaki et al. (1995) Neuron 14:651-659), which is described below.

- The present invention provides a method for identifying a  $\gamma$ -secretase inhibitory
- 25 substance wherein a compound which is known to act as a  $\gamma$ -secretase inhibitor (the substrate accumulating compound) is applied to a cell line that is expressing  $\beta$ -APP, thus allowing  $\beta$ - and  $\alpha$ -secretase processing of  $\beta$ -APP to occur in the absence of  $\gamma$ -secretase

- processing. This results in the accumulation of C-terminal fragments of  $\beta$ -APP which serve as a substrate for  $\gamma$ -secretase processing. The known  $\gamma$ -secretase inhibitor is then removed and replaced by a test substance. The ability of the test substance to inhibit  $\gamma$ -secretase activity can then be determined by quantifying the conversion of the
- 5 C-terminal fragments of  $\beta$ -APP to  $\beta$ -amyloid peptide.

- The method of the invention can be further understood with reference to the schematic representation of Fig. 1. As illustrated,  $\alpha$ - and  $\beta$ -secretase cleavage of  $\beta$ -APP in the presence of a substrate accumulating compound gives rise to the accumulation of
- 10 four C-terminal fragments due to the existence of two  $\alpha$ -processing sites and two  $\beta$ -processing sites. The shaded segments in the two C-terminal fragments produced by  $\beta$ -secretase cleavage represent the  $\beta$ -amyloid peptide segment. The shaded segments in two C-terminal fragments produced by  $\alpha$ -secretase cleavage represent a non-amyloidogenic fragment of the  $\beta$ -amyloid peptide. In the specific embodiment of the
- 15 invention illustrated in Fig. 1, the substrate accumulating compound is the compound MDL 28,170, which has the following formula (Medhi S. (1991) Trends Biochem. Sci. 16:150-153; Higaki et al. (1995) Neuron 14:651-659).



However, any compound which is known to accumulate substrate, whether by direct or indirect inhibition of  $\gamma$ -secretase activity, including  $\gamma$ -secretase inhibitor compounds identified using the method of this invention, can be employed as the substrate accumulating compound provided it has reversible activity.

5

The substrate accumulating compound is removed and replaced by a test substance. The processing (or absence of processing) of C-terminal fragments by  $\gamma$ -secretase cleavage is then monitored by quantitating  $\beta$ -APP carboxyl-terminal fragments in the cells,  $\beta$ -amyloid peptide in the medium or both. Graph (a) of Fig. 1

10 illustrates a typical result when the test compound exhibits no  $\gamma$ -secretase inhibitory activity. The presence of C-terminal fragments diminishes and the presence of  $\beta$ -amyloid peptide increases as  $\gamma$ -secretase processing occurs. Graph (b) illustrates a typical result when the test compound exhibits strong  $\gamma$ -secretase inhibitory activity. Little or none of the C-terminal fragments are converted to  $\beta$ -amyloid due to the inhibition of  $\gamma$ -secretase  
15 activity.

#### Cultured Cells

The assay method may employ primary or cultured cell lines of any type from any  
20 mammalian species. Since  $\beta$ -amyloid peptide is normally produced only at low levels in cultured mammalian cells, a preferred embodiment is to use a cell line which has been transfected with a  $\beta$ -APP encoding cDNA or gene operably linked to a promoter which is capable of effecting its expression in the host cell line, resulting in increased levels of the  $\beta$ -amyloid peptide. Standard vectors for recombinant mammalian cell expression  
25 (available from commercial sources such as InVitrogen or Stratagene) of the exogenous  $\beta$ -APP DNA are suitable such as those, for example, using the  $\beta$ -actin or cytomegalovirus promoters combined with a selectable drug marker such as neomycin or zeomycin.

Standard methods can be used for introducing exogenous DNA into the cell such as lipofectamine, calcium phosphate or electroporation. Isolation of single cell clones carrying the recombinant DNA is performed by standard methods usually making use of a drug-resistance gene harbored on the expression plasmid. These methods are described

- 5 in detail by the commercial manufacturer of the expression plasmid selected. In a preferred embodiment, a Chinese hamster ovary (CHO) cell line is used in conjunction with an expression plasmid in which a  $\beta$ -APP695 encoding cDNA is driven by a cytomegalovirus promoter and which possesses the neomycin-resistance gene. The expression plasmid is purchased from a commercial source, such as InVivoGen.
- 10 Lipofectamine, obtained from Gibco BRL, is used to introduce the DNA plasmid into the CHO cells and the drug G418 to select the CHO cells harboring the plasmid. Manufacturers directions are applied for the use of lipofectamine and for drug selection of cells with expression plasmid.

15

## Antibodies

- Antibodies to the  $\beta$ -amyloid peptide and to the carboxyl-terminus of  $\beta$ -APP are useful for analysis, isolation and quantitation of  $\beta$ -APP carboxyl-terminal fragments and  $\beta$ -amyloid peptide in the assay of the invention. To generate such antibodies, synthetic or
- 20 natural peptides can be used for the generation of the specific antibodies. Synthetic peptides are preferred since they can be used to raise antibodies to select regions of  $\beta$ -amyloid or  $\beta$ -APP and because of their general ease of preparation. Synthetic peptides are prepared by either the phosphotriester method as described by Edge et al. (1981) Nuc. Acids Res. 292:756 and Duckworth et al. (1981) Nuc. Acids Res. 9:1691 or the
- 25 phosphoramidite method as described by Beaucage and Caruthers (1981) Tet. Lett. 22:1859 and Matteucci and Caruthers (1981) J. Am. Chem. Soc. 103:3185, and can be prepared using commercially automated peptide synthesizers.

The synthetic or natural peptides may be conjugated to a carrier protein for animal immunization or they may be used as prepared. In a preferred embodiment, a synthetic peptide is coupled to a carrier such as keyhole limpet hemocyanin and to immunize rabbits according to standard methods. Similarly, the peptide conjugate can be used to immunize mice to prepare monoclonal antibodies. Techniques for raising monoclonal antibodies using B-lymphocytes from the spleen of the immunized mouse fused with an immortalized myeloma cell line are well known in the art. Selection, isolation, and characterization of the monoclonal hybridoma lines may be carried out by standard methods.

To prepare antibodies specifically to  $\beta$ -amyloid, it is preferred to use synthetic peptides conjugated to a carrier. Preferred peptides correspond to residues 1-13, 1-28, or 1-40 of  $\beta$ -amyloid. To prepare antibodies specific to the carboxyl-terminal cytoplasmic domain of  $\beta$ -APP, synthetic peptides have been demonstrated to yield reagents with the desired features. Peptides of 20 to 30 residues in length corresponding to sequences spanning residues 713 to 770 of the 770 amino acid  $\beta$ -APP isoform are preferred. One specific peptide consisting of residues 723-749 of  $\beta$ -APP 770 (or 704-730 of  $\beta$ -APP 751) has been effective (Higaki et al. (1995) Neuron 14:651-659). Preferably, rabbits or mice are immunized with the peptide conjugated to a carrier such as keyhole limpet hemocyanin. Both  $\beta$ -APP carboxyl-terminal and  $\beta$ -amyloid antibodies may be characterized for reactivity against their respective cognate immunogen in an enzyme-linked immunoadsorption sandwich assay. Further characterization of the polyclonal or monoclonal antibodies for the ability to react with native  $\beta$ -amyloid can be conducted using a variety of techniques known to the art, including, for example, immunoprecipitation of radiolabeled products, Western blot analysis, radioimmunoassay, or immunodisplacement assay.

### Identification of $\beta$ -Amyloid Inhibitors

- The method of the invention can be employed to determine the  $\gamma$ -secretase
- 5 inhibitory activity of any test substance. Preferred candidates for testing are substances which have previously been identified as inhibitors of  $\beta$ -amyloid formation.

- Inhibitors of  $\beta$ -amyloid production may be identified by several methods such as by application of compounds to cultured cells, semi-intact or cells whose membranes
- 10 have been rendered permeable that produce  $\beta$ -amyloid, and measuring the reduction of  $\beta$ -amyloid over a period of hours, preferably 1-8 hours. Measurement of  $\beta$ -amyloid may be made by immunoaffinity reagents using a variety of techniques, for example, immunoprecipitation, Western blot, enzyme-linked immunoadsorption or immunodisplacement assays. Active inhibitors are compounds which partially or fully
- 15 reduce levels of  $\beta$ -amyloid peptide. Active compounds may be of a wide array of chemical and/or protein structures.

### Assay Method for Direct $\gamma$ -Secretase Inhibitors

- 20 Cultured mammalian cells ( $\sim 10^5$  total cells) which produce and release  $\beta$ -amyloid peptide into the culture medium preferably at levels of 1-50 ng per ml may be used for the assay. In a preferred embodiment of the invention, cells in serum-free medium are treated with a pre-determined non-toxic but effective dose of substrate accumulating compound and cellular protein is simultaneously metabolically radiolabeled
- 25 for example by adding  $^{35}\text{S}$ -methionine (100-200  $\mu\text{Ci}$  per ml) to the serum-free medium. Preferably protein labeling is allowed to proceed for about 1 hour. After the treatment period, the medium containing the substrate accumulating compound and  $^{35}\text{S}$ -methionine

is removed, the cells are washed with an appropriate solution such as saline, and serum-free medium containing the agent to be tested is applied. Ideally, the test substance has been previously identified as an active inhibitor of  $\beta$ -amyloid formation. Preferably, the test substance is allowed to remain in contact with the cells for about 1 to 6 hours, most preferably about 4 hours. Cells and medium are harvested and each is analyzed. All of the cells and medium may be harvested at once at the end of the treatment period or aliquots may be harvested at various times during the treatment in order to provide a time course of conversion of  $\beta$ -APP carboxyl terminal fragments to  $\beta$ -amyloid peptide.

- 10 The harvested cells may be lysed to enable protein extraction by any of a number of standard methods but treatment with mild detergent is preferred. Protein can be extracted from the lysed cells by the method of Gabuzda et al. (1991) *J. AIDS* 4:34-40 or by other conventional methods. An aliquot of the protein extracted from the cells is reacted with a  $\beta$ -APP cytoplasmic domain-specific antibody to measure the levels of
- 15 carboxyl-terminal fragments present after treatment with the test substance. Immunoprecipitation is a preferred method of analysis. The immunoaffinity purified  $\beta$ -APP fragments are analyzed by polyacrylamide gel electrophoresis. Presence or absence of  $\beta$ -APP carboxyl-terminal fragments in the sample treated with the test substance is assessed by comparing the levels to that in cells having no substrate
- 20 accumulating compound or test substance added, as well as to cells continuously treated with only the substrate accumulating compound. Quantitation may be made by direct measurement of radioactivity present in the gel region of  $\beta$ -APP fragments or indirectly by computer integration of the exposed regions from the radiolabeled fragments. Quantitation of the  $\beta$ -APP carboxyl-terminal fragments in the cells treated with the test
- 25 substance, relative to the amounts present in the controls indicates the ability of the test substance to inhibit cleavage of the  $\beta$ -APP carboxyl-terminal fragments by  $\gamma$ -secretase.

These results, taken alone or in combination with the quantitation of  $\beta$ -amyloid in the cell culture medium, *infra.*, can be used to identify direct inhibitors of  $\gamma$ -secretase.

- The collected medium may be assessed for levels of  $\beta$ -amyloid protein, which is
- 5 another indicator of the conversion of accumulated  $\beta$ -APP carboxyl-terminal fragments by  $\gamma$ -secretase cleavage. It is known that, after  $\beta$ -amyloid protein is formed in the cell, it is immediately released into the extracellular environment (Haass et al. (1992) Nature 359:322-325; Seubert et al. (1992) Nature 359:325-327; Shoji et al. (1992) Science 258:126-129; Busciglio et al. (1993) Proc. Natl. Acad. Sci. USA 90:2092-2096;
- 10 Vigo-Pelfrey et al. (1993) J. Neurochem. 61:1965-1968; Tabaton et al. (1994) Biochem. Biophys. Res. Commun. 200:1598-1603; Teller et al. (1996) Nature Med. 2:93-95), in this case, the cell culture medium. An aliquot of medium is reacted with  $\beta$ -amyloid-specific antibodies, immunoprecipitated and analyzed by polyacrylamide gel analysis. Alternatively, an aliquot of the medium is electrophoresed on polyacrylamide gel and
- 15 then Western blotted. The medium can also be analyzed by an enzyme-linked immunoadsorption or immunodisplacement assay. The preferred mode of analysis is to use immunoprecipitation. Quantitation of  $\beta$ -amyloid can be made by comparing levels to cells in which the no substrate accumulating compound and agent were added and to cells persistently treated with only the substrate accumulating compound. Quantitation may be
- 20 made by direct measurement of radioactivity present in the gel region with  $\beta$ -APP fragments or indirectly by computer integration of the exposed regions from the radiolabeled fragments.

- Analysis of  $\beta$ -amyloid peptide and  $\beta$ -APP carboxyl-terminal fragments will
- 25 indicate whether an agent is a direct  $\gamma$ -secretase inhibitor or not. An agent which is not a direct  $\gamma$ -secretase inhibitor displays a conversion of radiolabeled accumulated substrate to



$\beta$ -amyloid peptide. An agent which is a direct  $\gamma$ -secretase inhibitor will display continued high levels of accumulated substrate and an absence of  $\beta$ -amyloid formation.

### EXAMPLE 1

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#### Identification of a Direct $\gamma$ -Secretase Inhibitor

##### A. Preparation of Assay Cell Line

A Chinese hamster ovary (CHO) cell line expressing recombinant human  $\beta$ -APP was used in the assay method. This cell line was prepared with the 695 amino acid  $\beta$ -APP isoform cDNA and a commercially available expression plasmid from InVitrogen. The expression plasmid, pcDNA3, has an immediate early gene cytomegalovirus promoter and a neomycin-resistance gene. The expression plasmid has the necessary sequences for bacterial replication plus an ampicillin resistance gene to allow for selection of bacteria containing plasmids. A ~2.3 kilobase pair SmaI-XmnI fragment of the  $\beta$ -APP cDNA which contains the entire coding region was cloned into the polylinker region of the plasmid. Bacterial clones were selected and evaluated for those possessing the  $\beta$ -APP cDNA in a sense orientation. Standard methods, including DNA sequence analysis, were used to isolate and characterize the appropriate expression construct. CsCl banded plasmid DNA was made and used to transfect wild-type CHO cells. Approximately 10  $\mu$ g of intact plasmid DNA was mixed with 50  $\mu$ l of Lipofectamine mix obtained from BRL and placed on a monolayer of  $\sim 5 \times 10^6$  CHO cells in a 10 cm dish. Roughly 72 hours after application of the DNA to the cells, 1 mg/ml G418 was added to the medium (a 1:1 mixture of Dulbecco's minimum essential medium and Coon's F12 medium with 10% fetal calf serum) to select the CHO cells harboring the plasmid DNA. After 2-4 weeks, G418 resistant CHO colonies were evident. Dilution plating was employed to isolate single cell clones.

- Once single cell CHO clones were expanded they were assessed for levels of  $\beta$ -APP and  $\beta$ -amyloid peptide. This was achieved by metabolically radiolabeling a collection of random clones with 150  $\mu$ Ci/ml  $^{35}$ S-methionine in serum-free medium for
- 5 6 hours. The medium from each clone was harvested and the clone monolayers were washed with saline then prepared according to the method of Gabuzda et al. (1991) *J. AIDS* 4:34-40. To measure  $\beta$ -APP and  $\beta$ -amyloid levels, immunoprecipitation was used. For  $\beta$ -APP, each cell lysate was diluted to 5 ml with RIPA buffer (7 ml: 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100), was
- 10 precleared with 4  $\mu$ l normal rabbit serum and 100  $\mu$ l protein A-Sepharose by rocking at 23°C for 2.5 hours or 4°C overnight followed by centrifugation at low speed. Thirty  $\mu$ l of a polyclonal rabbit antiserum, BC1, raised to a synthetic peptide corresponding to the  $\beta$ -APP cytoplasmic domain (residues 704-730 of  $\beta$ -APP 751) and 100  $\mu$ l protein A-Sepharose were added to the lysate mixture after which the immunoprecipitation was
- 15 incubated at 23°C for 2.5 hours. The mixture was centrifuged at low speed and the pellet was washed twice with 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, twice with 50 mM Tris-HCl (pH 7.5) 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and twice with 10 mM Tris-HCl (pH 7.5). The pellet was resuspended in 100  $\mu$ l SDS Laemmli sample reducing buffer and 20  $\mu$ l were applied to a 8% SDS
- 20 polyacrylamide gel for electrophoresis. Gel autoradiograms were developed and quantitative densitometry of the 105-130 kDa  $\beta$ -APP protein bands was made using a Phosphorimager from Molecular Dynamics. Immunoprecipitation of the medium was made by clarification of the medium by low speed centrifugation followed by preclearing with 4  $\mu$ l normal rabbit serum and 100  $\mu$ l protein A-Sepharose using the conditions
- 25 described for the cell lysates. Forty five  $\mu$ l of a rabbit polyclonal antiserum raised to synthetic  $\beta$ -amyloid 1-40 was added to the medium sample and was treated in an identical manner as the  $\beta$ -APP immunoprecipitation. The final pellet was resuspended in 50  $\mu$ l

Laemmli SDS reducing buffer and applied to a 16.5% Tris-Tricine-SDS polyacrylamide gel. Gel autoradiograms were developed and quantitative densitometry of the ~4 kDa  $\beta$ -amyloid protein band was made using a Phosphorimager from Molecular Dynamics. A comparative analysis of the clones identified a single clone with the highest levels of both  $\beta$ -APP and  $\beta$ -amyloid. This clone was selected as the assay cell line.

### B. $\beta$ -APP Substrate Accumulating Compound

A chemical compound was identified which inhibits  $\beta$ -amyloid formation when applied to cultured mammalian cells and which causes the accumulation of the immediate  $\beta$ -APP proteolytic precursors to the  $\beta$ -amyloid peptide (Higaki et al. (1995) Neuron 14:651-659). This compound, MDL 28,170, is a dipeptide aldehyde calpain inhibitor (Medhi S. (1991) Trends Biochem Sci. 16:150-153; Higaki et al. (1995) Neuron 14:651-659). Its structure is described above. Fig. 2 is an autoradiograph showing the effects of the substrate accumulating compound, MDL 28,170, in which  $\beta$ -amyloid formation is blocked and a concomitant increase in C-terminal  $\beta$ -APP fragments is observed (upper panel). The gel pattern on the left, designated by (+) indicates application of the substrate accumulating compound and the gel pattern on the right, designated (-) is the untreated control. The upper panel shows that the cell lysate from the cells cultured in the presence (+) of MDL 28,170 for 4 hours contains substantial amounts of accumulated C-terminal fragments, whereas the lysate from the untreated control cells exhibit only normal levels of C-terminal fragments. The lower panel shows that the medium from the cells cultured in the presence (+) of MDL 28,170 for 4 hours does not contain detectable  $\beta$ -amyloid, whereas the medium from the untreated control cells contains  $\beta$ -amyloid peptide.

### C. Identification of $\gamma$ -Secretase Inhibitory Candidates

Agents which block  $\beta$ -amyloid formation were identified by applying random chemical agents to the CHO assay cell line and measuring  $\beta$ -amyloid production. Agents X and Y were identified as active  $\beta$ -amyloid inhibitors in the following manner.

Monolayers of CHO cells ( $\sim 10^6$  cells per well) plated in a 6 multi-well plate in a 1:1

- 5 mixture of Dulbecco's minimum essential medium and Coon's F12 medium with 10% fetal calf serum were grown for about 24 hours after which the cell monolayers were washed twice with saline then placed in 2 ml of Delbecco's minimal essential medium 21 containing 150  $\mu$ Ci/ml  $^{35}$ S-methionine. Individual agents were applied to the cells at 25  $\mu$ M for 4 hours. After the treatment period, the medium was harvested and assayed
- 10 for  $\beta$ -amyloid peptide using immunoprecipitation with a  $\beta$ -amyloid-specific antiserum as detailed in the previous section. Both agents X and Y displayed complete inhibitory activity for  $\beta$ -amyloid production at 25  $\mu$ M as well at concentrations of 50  $\mu$ M and 100  $\mu$ M. No gross toxicity was detected based on morphology of the cells, determination of general protein radiolabeling by examining  $\beta$ -APP in the cell lysates as described
- 15 above, and by using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (Hansen et al. (1989) J. Immunol. Methods 119:203-210).

#### D. Conducting the Assay

- 20 The CHO cell line was plated into a 6 multi-well plate with  $\sim 10^6$  cells per well. Approximately 24 hours after plating the cell monolayers were washed twice with saline and 2 ml of serum-free medium (Dulbecco's minimum essential medium 21) containing 100  $\mu$ Ci/ml  $^{35}$ S-methionine and 200  $\mu$ M of the substrate accumulating compound, MDL 28,170 (Higaki et al. (1995) Neuron 14: 651-659) were added. Radiolabeling and
- 25 compound treatment was for 1 hour. After this period, the cell monolayers were washed free of label and substrate accumulating compound by rinsing the cells three times with saline. Two ml of fresh serum-free medium containing 25  $\mu$ M agent X was added to the

monolayers and then incubated for 4 hours. At the end of the treatment period, medium and cells were harvested and analyzed for  $\beta$ -amyloid and  $\beta$ -APP carboxyl-terminal fragments, respectively, using immunoprecipitation followed by polyacrylamide gel electrophoresis and quantitative densitometry of the gel bands, as described in Section A

5 above. Results of the densitometry scans are presented graphically in Fig. 3, in which the results of the scans are reported in arbitrary pixel units (A.U.) generated by the computerized scanning. The upper panel demonstrates the lack of  $\beta$ -amyloid production during the treatment period with agent X indicating inhibition of  $\beta$ -amyloid formation. The lower panel of Fig. 3 reveals the lack of utilization of radiolabeled  $\beta$ -APP carboxyl-

10 terminal substrate, indirectly confirming inhibition of  $\beta$ -amyloid production by direct inhibition of  $\gamma$ -secretase.

## EXAMPLE 2

### Identification of an Inactive Direct $\gamma$ -Secretase Inhibitor

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Agent Y was tested in the assay method in an identical manner as agent X. In Fig. 4, the upper panel reveals that agent Y, while it is an active inhibitor of  $\beta$ -amyloid formation (Example 1; Section C), is not a direct  $\gamma$ -secretase inhibitor since the agent did not inhibit  $\beta$ -amyloid formation: radiolabeled  $\beta$ -amyloid was found present in the culture

20 medium. The lower panel of Fig. 4 shows that the radiolabeled  $\beta$ -APP carboxyl-terminal substrates are depleted indirectly confirming the lack of  $\gamma$ -secretase inhibition by agent Y. Agent Y was next reconfirmed as an active general  $\beta$ -amyloid inhibitor according to the procedure described in Example 1 Section C.

What is claimed is:

1. A method for identifying a direct acting  $\gamma$ -secretase inhibitor which comprises:

5

(A) culturing a cell line which expresses a DNA sequence encoding  $\beta$ -APP and which is capable of producing  $\beta$ -amyloid peptide in contact with a substrate accumulating compound, thereby causing the accumulation of  $\beta$ -APP carboxyl-terminal fragments in the cells;

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(B) removing the substrate accumulating compound;

(C) contacting the cell line with a test substance; and

15

(D) determining the  $\gamma$ -secretase inhibitory activity of the test substance by quantifying the amount of carboxyl-terminal fragments of  $\beta$ -APP in the cells and/or quantifying the amount of  $\beta$ -amyloid peptide in the cell culture medium after the cell line has been in contact with the test substance.

20

2. A method as claimed in claim 1, further comprising the step of labeling the cellular protein in the cell culture with a quantifiable label.

25

3. A method as claimed in claim 2, wherein the quantifiable label is a radioactive label.

4. A method as claimed in claim 3, wherein the cellular protein is labeled by culturing the cell line in a medium containing the substrate accumulating compound and a radioactively labeled amino acid.

5. A method as claimed in claim 3, wherein the radioactive label is <sup>35</sup>S-methionine.

6. A method as claimed in claim 1, wherein the carboxyl-terminal fragments of  $\beta$ -APP in the cells are quantified by:

(A) lysing the cells and extracting the cellular protein;

(B) immunoprecipitating the carboxyl-terminal fragments of  $\beta$ -APP from the cellular protein;

(C) subjecting the carboxy-terminal fragments of  $\beta$ -APP to polyacrylamide gel electrophoresis; and

(D) quantifying the carboxy-terminal fragments of  $\beta$ -APP on the gel.

7. A method as claimed in claim 6, wherein the carboxyl-terminal fragments of  $\beta$ -APP are immunoprecipitated with an antibody specific to the cytoplasmic domain of  $\beta$ -APP.

8. A method as claimed in claim 6, wherein the cellular protein is radiolabeled and the carboxy-terminal fragments of  $\beta$ -APP are quantified by

measurement of radioactivity in the regions of the gel corresponding to the carboxyl-terminal fragments of  $\beta$ -APP.

9. A method as claimed in claim 4 wherein the medium containing the  
5 substrate accumulating compound is removed after it has been in contact with the cell culture for a period of time and is replaced by cell culture medium containing the test substance.

10. A method as claimed in claim 9, wherein the medium containing the  
10 substrate accumulating compound and radioactive labeled amino acid is removed after it has been in contact with the cell culture for a period of about one hour.

11. A method as claimed in claim 1, wherein the cells are contacted with the  
test substance for a period of from 1 to 6 hours.

15

12. A method as claimed in claim 1, wherein the cells are contacted with the  
test substance for a period of about 4 hours.

13. A method as claimed in claim 1, wherein the cells are mammalian cells  
20 which have been transfected with cDNA or a gene encoding  $\beta$ -APP under the control of a promoter which is capable of effecting the expression of the cDNA or gene in the host cell.

14. A method as claimed in claim 13, wherein the cells are intact cells.

25

15. A method as claimed in claim 13, wherein the cells have cell membranes  
which have been ruptured or rendered permeable.



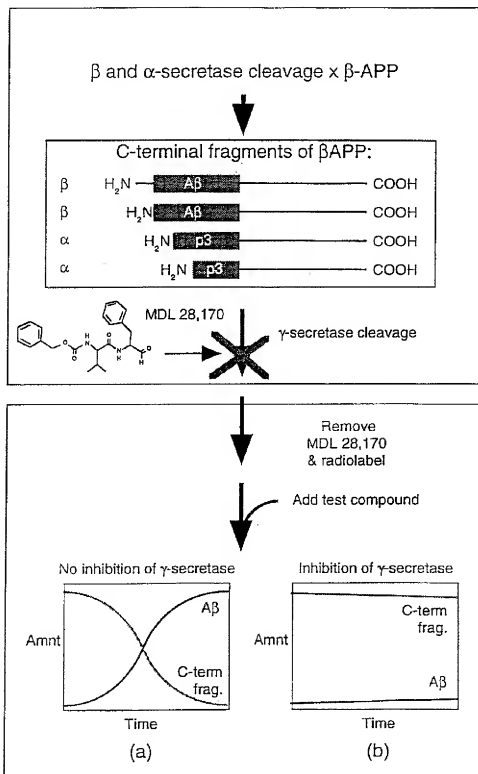


FIG.1

2/4

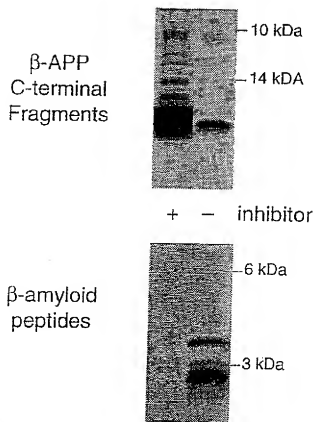


FIG. 2

3/4

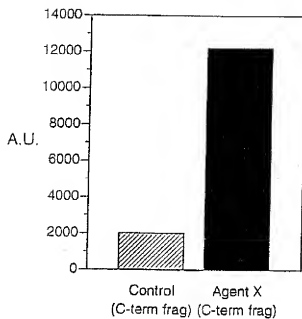
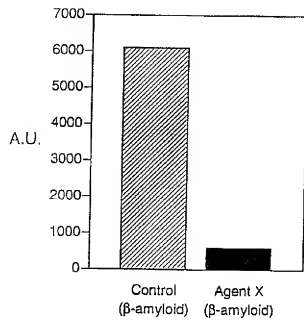


FIG. 3

4/4

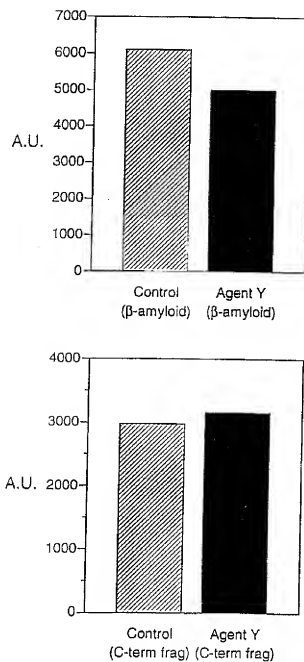


FIG. 4

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/16988

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/50 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category \* Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

Y J. HIGAKI, ET AL.: "inhibition of beta-amyloid formation identifies proteolytic precursors and subcellular site of catabolism." NEURON, vol. 14, March 1995, pages 651-659, XP002053880 see abstract see page 654, left-hand column, line 7-12 see page 657, left-hand column, line 4 - line 26

1-15

Y EP 0 569 777 A (MILES INC) 18 November 1993 see claim 19

1-15

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☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex

### \* Special categories of cited documents:

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Date of the actual completion of the international search

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13/02/1998

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Hoekstra, S

## INTERNATIONAL SEARCH REPORT

 International Application No  
 PCT/US 97/16988

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